et al., 2001). Another question arising is the release of Spitz and TGFα by different mechanisms. The Freeman group notes that several vertebrate Rhomboid and TGFα-related proteins are yet to be fully characterized, suggesting that the current designation of Spitz as the *Drosophila* homolog of TGFα may be revised.

Nonetheless, several conclusions can be drawn at this time. First, the utility of RIP is bound to expand to additional biological processes and substrates, and additional proteins with I-CliP activity will likely be discovered. Second, the fact that the functional domain of the first RIP substrates were all within the cytoplasm may be just a coincidence. This is of particular importance as it complicates the analysis of RIP substrates whose biological activity is unknown, such as APP. APP may resemble Spitz more than it does Notch. Third, the regulation of receptor tyrosine kinase and their ligands has grown in complexity as a mysterious function of Rhomboid is revealed.

The one bit of bad news—not only do we need to account for all gene products and splice variants, it is now crystal clear we need to pay close attention to protein fragments as well.

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Live Stripping of Clathrin-Coated Vesicles

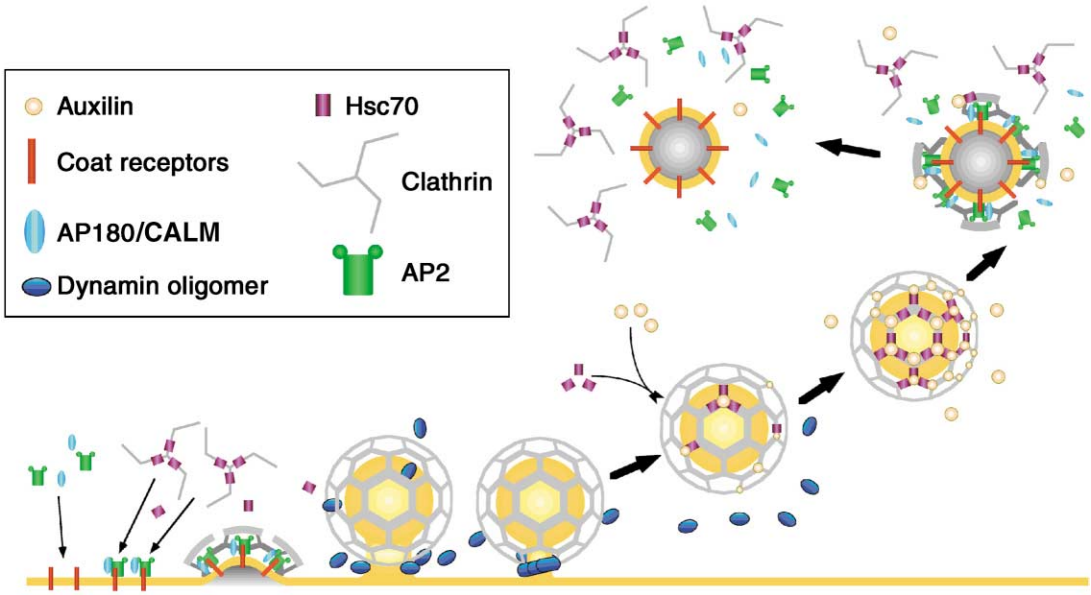
Vesicle budding requires recruitment of a coat, which must then be removed to allow fusion with the target compartment. In vitro assays have implicated Hsc70 and auxilin family members as key players in clathrin-coated vesicle uncoating. New in vivo studies now show that this is indeed the case and reveal additional functions of the Hsc70/auxilin complex.

Dynamic interactions between anatomically distinct membrane compartments rely on the continuous generation of transport vesicles. Vesicle budding requires recruitment of a coat that provides mechanical support for membrane bending and fission, and helps to concentrate specific cargos. After fission, the coat must be shed to allow fusion of the vesicle with the target membrane (Figure 1).

Clathrin-coated vesicles (CCVs), which mediate transport from the plasma membrane and from internal compartments, are the most thoroughly investigated transport vesicles. Current models propose that clathrin coat recruitment starts with the binding of adaptors to the membrane. This binding involves interactions with both membrane proteins and lipids, primarily phosphoinositides. Bound, oligomerized adaptors then act as a template for the recruitment of clathrin triskelia (Figure 1). Via a series of still poorly understood intermediate stages, a deeply invaginated clathrin-coated bud is formed and is eventually excised from the membrane.

Unlike coat deposition, stripping of the clathrin coat from CCVs to form free vesicles requires energy. Almost 20 years ago, an ATPase with the unusual property of uncoating CCVs in vitro was purified from brain cytosol (Schlossman et al., 1984). This protein was subsequently identified as a constitutively and ubiquitously expressed member of the Hsp70 chaperone family and thus called Hsc70 (heat-shock cognate protein 70 kDa). Since then, a wealth of information has accumulated on its function in vitro. Hsc70 has an evolutionarily conserved N-terminal ATPase domain. The activity of this domain is required for function in the uncoating reaction, and it regulates the affinity of a central binding pocket for clathrin. ATP hydrolysis is the rate-limiting step of the reaction. Although Hsc70 increases its catalytic activity considerably upon binding to clathrin, this is not sufficient to drive uncoating efficiently. The catalytic activity is dramatically enhanced by specific co-chaperones—members of the DnaJ protein family—which are able to speed up the reaction more than an order of magnitude. Two such proteins have been linked to clathrin uncoating: the brain specific auxilin (auxilin 1) and the ubiquitously expressed auxilin 2 (also referred to as GAK). DnaJ proteins bind Hsc70 by means of a highly conserved 80 amino acid J domain. They also bind the Hsc70 substrate clathrin and the clathrin adaptors AP-1 and AP-2. Based on these and other data, it has been proposed that auxilins recruit Hsc70 to the coat and then trigger the catalytic activity by DnaJ-mediated interactions. However, kinetic considerations about clathrin uncoating and the observation that clathrin triskelia are released with Hsc70 tightly bound have suggested additional chaperone functions in endocytosis for Hsc70, including stabilization of soluble clathrin to avoid spontaneous polymerization and clathrin priming for rebinding to membranes after uncoating (Jiang et al., 2000).

Until very recently, there was no in vivo evidence of an Hsc70/auxilin function in uncoating. However, this situation is rapidly changing. Dominant-negative interference studies in mammalian cells have shown that Hsc70 not only takes part in the uncoating reaction in vivo but that it also plays a general role in clathrin dynamics (Newmyer



A Model for Clathrin-Mediated Endocytosis

Clathrin adaptors are recruited by protein and lipid signals. Then, clathrin assembly proceeds to the formation of a coated pit, which is finally excised to generate a free-coated vesicle. Hsc70 and auxilin take part to the uncoating reaction by binding to clathrin. ATP hydrolysis by Hsc70 drives the disassembly of polymerized clathrin. Although auxilin is released during this process, Hsc70 remains bounded to free clathrin to perform additional functions, including avoiding clathrin-spontaneous polymerization and priming it for adaptor complex-mediated recruitment to membranes.

and Schmid, 2001). Inactivation of yeast auxilin (Swa2) results in defects similar to clathrin mutants (Gall et al., 2000) and in accumulation of CCVs (Pishvaee et al., 2000). RNA interference of nematode auxilin blocks receptor-mediated endocytosis in oocytes and alters clathrin dynamics in somatic cells (Greener et al., 2001). However, owing to the intrinsic limitations of the RNA interference approach in neurons, the nematode studies were not able to address the important question of clathrin uncoating in brain.

Recycling of synaptic vesicle components after secretion of the neurotransmitter is necessary to stabilize synaptic activity during prolonged stimulation. Several lines of evidence suggest that this membrane trafficking pathway relies heavily on clathrin-mediated endocytosis. A paper from Morgan et al. (2001, October 25 issue) in a recent issue of *Neuron* provides definitive evidence for an essential role of the Hsc70/auxilin complex in synaptic activity. Using a combination of in vitro assays and microinjection experiments in squid giant synapses, the authors were able to dissect the contributions of the various interaction domains of auxilin (the J domain) and Hsc70 (the ATPase domain and the substrate binding domain) in uncoating and synaptic function. Of particular interest is the identification of the HPD tripeptide within auxilin's J domain as the key binding site for Hsc70, as this recognition sequence is evolutionarily conserved even in prokaryotes. Furthermore, this paper provides the first direct evidence for an essential role of clathrin uncoating in efficient synaptic vesicle recycling and suggests that CCV stripping may represent the crucial rate-limiting step of this process. As the requirement for uncoating occurs during low frequency stimulation, the authors clearly demonstrate that clathrin-dependent synaptic vesicle endocytosis is not only required for intense synaptic activity.

Although a great deal of information has now been collected on the molecular events underlying clathrin uncoating, several key questions remain. Why does the uncoating machinery apparently act only on free-coated vesicles but not on the nascent coat? How are clathrin adaptor complexes released from the membrane, as Hsc70 is required but not sufficient for this process in cell-free assays (Hannan et al., 1998)? The synaptojanin-dependent dephosphorylation of phosphoinositides may play a role in this, as suggested by the accumulation of CCVs at the synapses of synaptojanin-deficient nematodes and mice. Interestingly, adaptors can be released independently of clathrin (Hannan et al., 1998) and phosphoinositides may interact with both auxilin 1 and auxilin 2 by means of their PTEN-like domains. Another important question relates to the role played by phosphorylation in the uncoating reaction. Is there a cycle of phosphorylation/dephosphorylation to regulate uncoating, as shown for the recruitment of endocytic proteins in early steps of membrane recycling? It is interesting that auxilin 2 has a kinase domain homologous to yeast Ark/Prk kinases, which function in actin regulation and endocytosis, and that it phosphorylates AP-1 and AP-2 medium chains in vitro (Umeda et al., 2000). Last but not least, genetic analysis of Hsc70-4 function in flies has highlighted an unexpected link to neurotransmitter exocytosis, probably mediated by cysteine string proteins (Bronk et al., 2001). Morgan et al. have data suggesting that this is also true in squid synapses, as they observe a block in exocytosis without complete vesicle depletion.

The boost given to the field by the advent of the in vivo era of clathrin uncoating promises to test these and other open questions in the near future.

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